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Injury-Induced Class 3 Semaphorin Expression in the Rat Spinal Cord

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In this study we evaluate the expression of all members of the class 3 semaphorins and their receptor components following complete transection and contusion lesions of the adult rat spinal cord. Following both types of lesions the expression of all class 3 semaphorins is induced in fibroblast in the neural scar. The distribution of semaphorin-positive fibroblasts differs markedly in scars formed after transection or contusion lesion. In contusion lesions semaphorin expression is restricted to fibroblasts of the meningeal sheet surrounding the lesion, while after transection semaphorin-positive fibroblast penetrate deep into the center of the lesion. Two major descending spinal cord motor pathways, the cortico- and rubrospinal tract, continue to express receptor components for class 3 semaphorins following injury, rendering them potentially sensitive to scar-derived semaphorins. In line with this we observed that most descending spinal cord fibers were not able to penetrate the semaphorin positive portion of the neural scar formed at the lesion site. These results suggest that the full range of secreted semaphorins contributes to the inhibitory nature of the neural scar and thereby may inhibit successful regeneration in the injured spinal cord. Future studies will focus on the neutralization of class 3 semaphorins, in order to reveal whether this creates a more permissive environment for regeneration of injured spinal cord axons.

Key Words: **collapsin-responsive mediator protein; contusion injury; corticospinal tract; neuropilin; plexin; regeneration; rubrospinal tract; semaphorin; spinal cord; transection.**

INTRODUCTION

Although some central nervous system (CNS) neurons have the intrinsic capacity to regenerate following injury (3), this regenerative potential is frustrated by the presence of outgrowth inhibitory molecules and the lack of sufficient trophic support in the microenvironment surrounding the injured axon stumps (4, 5, 26). Molecules associated with CNS myelin, like NI-250/nogo A (9, 57) and myelin-associated-glycoprotein (MAG) (40, 42), inhibit regrowth of injured neurites. Furthermore, enhanced expression of neurite outgrowth inhibiting extracellular matrix proteins, including chondroitin sulfate proteoglycans (CSPG) (12, 39, 59, 60) and tenascin (2, 32, 33, 36, 71), by reactive astrocytes and other scar-associated cells at the injury site contributes to regenerative failure of CNS axons (14, 53, 61–63).

Several axon guidance molecules, including members of the netrin, ephrin, slit, and semaphorin families, display chemorepulsive effects on specific populations of axons in the developing embryo (22, 27, 37, 69). Observations in genetically manipulated mice demonstrated that these proteins are involved in constraining axons to defined regions of the developing nervous system (7, 13, 16, 58, 67). We and others (18, 19, 37, 70) hypothesized that some of these chemorepulsive proteins could also participate in inhibiting regeneration in the adult injured mammalian CNS. Indeed fibroblasts and astrocytes in the core of a neural scar formed following traumatic brain injury express high levels of the repellents semaphorin 3A (Sema3A) (46, 47) and EphB3 (41), respectively. The continued expression of neuropilin-1, a component of the Sema3A receptor, by axotomized olfactory and dorsal root ganglion neurons, suggests that these axons are sensitive to scar-derived Sema3A (46).

The semaphorin family consists of secreted and membrane associated proteins, which all share a semaphorin domain of approximately 500 amino acids (29). Based on similarities in their semaphorin domain and differences in C-terminal characteristics semaphorins have been subdivided into eight classes (1). Class 3 semaphorins are secreted vertebrate semaphorins, which mediate their repulsive effect via a receptor

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complex consisting of neuropilin-1 or neuropilin-2 (NP-1, NP-2) and a signal transducing part formed by class A plexins (PlxA1) (54, 64, 66). Antibody neutralization of NP-1 prevents Sema3A-induced growth cone collapse, showing that NP-1 is an essential protein of the functional receptor complex (15, 23, 28). NP-2 is required for Sema3B, Sema3C, and Sema3F function (11, 17, 65). Both *in vitro* and *in vivo,* semaphorin-induced biological responses can be suppressed by expression of dominant-negative plexins, indicating the essential role of plexins in semaphorin signaling (54, 64, 66). The intracellular signaling pathway of secreted semaphorins is largely unknown although collapsin-responsive mediator protein (CRMP-2) is required for Sema3A activity on dorsal root ganglion neurons (20), and activation of rho-like GTPases appears to be involved (25, 30, 55).

In this study we document the expression of all rodent class 3 semaphorins in relation to the failure of adult long descending spinal cord axons, e.g., cortico- and rubrospinal axons, to regenerate following spinal cord injury. Individual class 3 semaphorin genes were analyzed by *in situ* hybridization at different time points following two types of spinal cord lesions, complete spinal cord transection, and spinal cord contusion lesions. We find that all class 3 semaphorin family members are expressed at moderate-to-high levels in the fibroblast component of the developing neural scar at the spinal cord lesion site. Cortico- and rubrospinal tract (CST and RST, respectively) neurons continue to express receptor components for class 3 semaphorins and most descending spinal cord fibers were not able to penetrate the portion of the scar containing semaphorin expressing fibroblasts. The observation that semaphorin-positive scar tissue is selectively avoided by injured descending spinal cord axons suggests a significant contribution of class 3 semaphorins to regenerative failure of the CST and RST axons.

MATERIALS AND METHODS

Animals

Contusion lesions ($n = 16$; four animals per condition per time point) and retrograde tracing ($n = 2$) were performed in adult male Wistar rats (220 g; Harlan CPB-Zeist; The Netherlands). Control tissue was derived from adult male rats ($n = 4$). Complete spinal cord transections and fiber tracing ($n = 16$; 4 animals each time point) were performed on adult female Fischer rats (160–180 g; Charles River Laboratories, Raleigh, NC). The animals were housed in group cages in 12-h light/12-h dark cycle and food and water were available *ad libitum*. All surgical techniques were performed according to the local guidelines of the Experimental Animal Care Committee.

Surgical procedures

Spinal cord contusion lesion. Anesthesia and surgical procedures were performed as described previously (31). In short, animals were anesthetized, intubated, and artificially ventilated with a mixture of 1.75% isoflurane and O_2/N_2O (1:2). The spinal cord was exposed by bilateral laminectomy of thoracic vertebra 8, without disrupting the dura. The MASCIS-Impactor (21) with a 10-g rod was used to induce a 12.5- or a 25-mm contusive injury. Muscle and skin were sutured and the animals received 3 ml saline subcutaneously to compensate for blood loss during the surgical procedures. The bladder was emptied manually until sufficient autonomous emptying of the bladder returned. Animals were sacrificed at 14 and 28 days after surgery.

Complete spinal cord transection. Anesthesia and surgical procedures were performed as described previously (44). In short, the thoracic spinal cord was exposed and a laminectomy was performed at thoracic vertebra T8. A small incision was made in the dura mater, followed by a complete transection of the T9 spinal cord. The lesion site was covered with 0.15-mm-thick silicone sheeting (Specialty Manufacturing, Saginaw, MI), and the muscles and skin were sutured. Following surgery the rats received a subcutaneous injection of 10 ml lactated Ringers solution, and were allowed to recover in a warmed cage. Twice a day the bladder was emptied manually until bladder function returned. The rats were sacrificed 7, 14, 28, and 56 days after surgery.

Anterograde tracing. One week before the completely transected animals were sacrificed, the descending axon projections in the spinal cord were anterogradely labeled with biotinylated dextran amine (BDA; 10,000 MW; Molecular Probes, Eugene, OR). Four times 0.5 μl 10% BDA was injected at the T6–7 thoracic spinal cord level (in both dorsal and ventral horns). Animals that were analyzed at 7 days postinjury were injected with BDA immediately after the complete transection.

Retrograde tracing. Following exposure of the vertebral column, a laminectomy was performed at thoracic vertebra T7. Injections with Fluorogold (1 μl; 4% in 0.1% DMSO/H_2O; Molecular Probes) were performed in the dorsal half of the spinal cord, both dorsal horns one injection, to retrogradely trace the descending fibers. Seven days following injection the animals were sacrificed. Double staining for one of the receptor component mRNAs, NP-1, NP-2, PlxA1, or CRMP-2 and for Fluorogold was used to identify the corticospinal neurons.

Tissue Preparation

At the appropriate postoperative survival times animals were deeply anesthetized with Nembutal (0.125

ml/100 g; ip), transcardially perfused with 0.1 M phosphate buffer (PB; pH 7.4), and subsequently fixed by perfusion with 4% paraformaldehyde (PFA) in 0.1 M PB (pH 7.4). Brain and spinal cord were dissected and postfixed overnight in 4% PFA at 4°C. Overnight treatment with EDTA (4°C; 250 mM in PB) to enhance tissue penetration was followed by 24 h of cryoprotection by immersion in 25% sucrose in PB at 4°C. Tissue was rapidly frozen in dry-ice-cooled 2-methylbutane and stored in −80°C until use.

Thionine Staining

Cryosections (20 μm) were thaw-mounted on Superfrost microscope slides (Menzel Gläser, Germany) and immersed in thionine solution (0.5% thionine; 1% acetic acid). Sections were rinsed in water, subsequently dehydrated in graded series of ethanol, cleared in xylene, and coverslipped with Entellan (Merck).

In Situ Hybridization

Digoxigenin- (DIG, Boehringer Mannheim, Mannheim, Germany) labeled cRNA probes were generated by *in vitro* transcription with T7 RNA polymerase (Boehringer Mannheim), using linearized full-length templates (Sema3A (rat (19)), Sema3B, Sema3C, Sema3E, Sema3F, NP-2, and PlxA1 (mouse cDNA) were a gift from A.W. Püschel, and NP-1 was a gift from A. L. Kolodkin). *In situ* hybridization was performed as described previously (47). In short, cryosections (20 μm) were thaw-mounted on Superfrost microscope slides (Menzel Gläser) and postfixed with 4% paraformaldehyde in phosphate-buffered saline (PBS; pH 7.4). Following proteinase K (10 μg/ml in PBS, 10 min, room temperature (RT)) treatment and acetylation sections were prehybridized overnight at RT in hybridization solution (5× SSC; 50% formamide; 5× Denhardt's; 125 mg/ml baker's yeast tRNA). Hybridization at 60°C was performed overnight using 200 ng/ml heat-denatured digoxigenin-labeled cRNA probe in hybridization solution. Stringency washes (5× SSC, 5 min/60°C; 2× SSC, 1 min/60°C; 0.2× SSC/50% formamide, 30 min/60°C; 0.1× SSC, 5 min/RT) were followed by a blocking period of 1 h in blocking reagents (Boehringer Mannheim). Alkaline phosphatase-conjugated anti-DIG antibody (1:3000 in Tris-buffered saline (TBS), pH 7.5, 3 h, RT) incubation, followed by overnight incubation at RT with nitroblue tetrazolium/5-bromo-4-chloro-indolyl phosphate in TBS (pH 9.5) containing magnesium, led to visualization of the hybridized probe. The sections were mounted in glycerol or used for immunocytochemistry.

Class 3 members of the semaphorin family contain related sequences [0–59% identical nucleotides in the complete coding sequence and 48–64% identical nucleotides in the semaphorin domain (alignment performed with CLUSTAL program)]. The specificity of the *in situ* hybridization procedure was inferred from partially overlapping, but clearly distinct, distribution patterns of all probes hybridized to sections derived from noninjured rat brains (not shown). Sections taken from the spinal cord lesion area or from noninjured spinal cord hybridized with sense probes or without probe exhibited no hybridization signal.

Immunocytochemistry

Following *in situ* hybridization, immunocytochemistry was performed. After rinsing in 0.1 M TBS (pH 7.4) and blocking in TBS containing 0.1% Triton X-100 and 0.2% bovine serum albumin (BSA), sections were incubated overnight at 4°C with primary antibody in TBS. Washing in TBS and incubation for 1 h at RT with peroxidase-labeled secondary antibody (1:800 in TBS) was followed by anti-peroxidase antibody (1:1000 in TBS) incubation for 1 h at RT. Sections were briefly washed in TBS and rinsed in 0.05 M Tris–HCl, pH 7.6, and treated with 0.05% diaminobenzidine (DAB) and 0.003% H_2O_2 in 0.05M Tris–HCl, pH 7.6. Peroxidase reaction was stopped in TBS and sections were mounted in glycerol.

Antibodies

To characterize the class 3 semaphorin-expressing cells in the lesion site the following antibodies were used: anti-vimentin (vimentin is an intermediate filament present in a variety of cells, e.g., meningeal cells, fibroblasts, and reactive astrocytes; monoclonal; 1:20; Boehringer Mannheim), anti-fibronectin (marker for meningeal cells and fibroblasts; rabbit polyclonal; 1:40; Boehringer, Ingelheim, Heidelberg, Germany), anti-glial fibrillary acidic protein (GFAP; marker for mature astrocytes; 1:800; Dako, Glostrup, Denmark), and anti-S100 (marker for astrocytes and Schwann cells; 1:600; Dako). Anti-Fluorogold (1:5000, Chemicon International) was used to visualize the retrogradely transported Fluorogold in CST and RST neurons.

RESULTS

Development of the Spinal Cord Lesion Site

In order to study neural scar formation in the transected and contused rat spinal cord, we performed thionine staining. We analyzed three horizontal sections in each animal; each section was taken from a different dorsal–ventral level through the spinal cord lesion site.

Complete transection (Figs 1A, 1C, 1E, and 1G). In the first week postlesion blood has accumulated at the primary lesion site and signs of tissue loss were observed at the distal edge of the lesion. Along the blood clot an accumulation of infiltrated cells was observed (Fig. 1A). After 14 days the blood clot was cleared, and

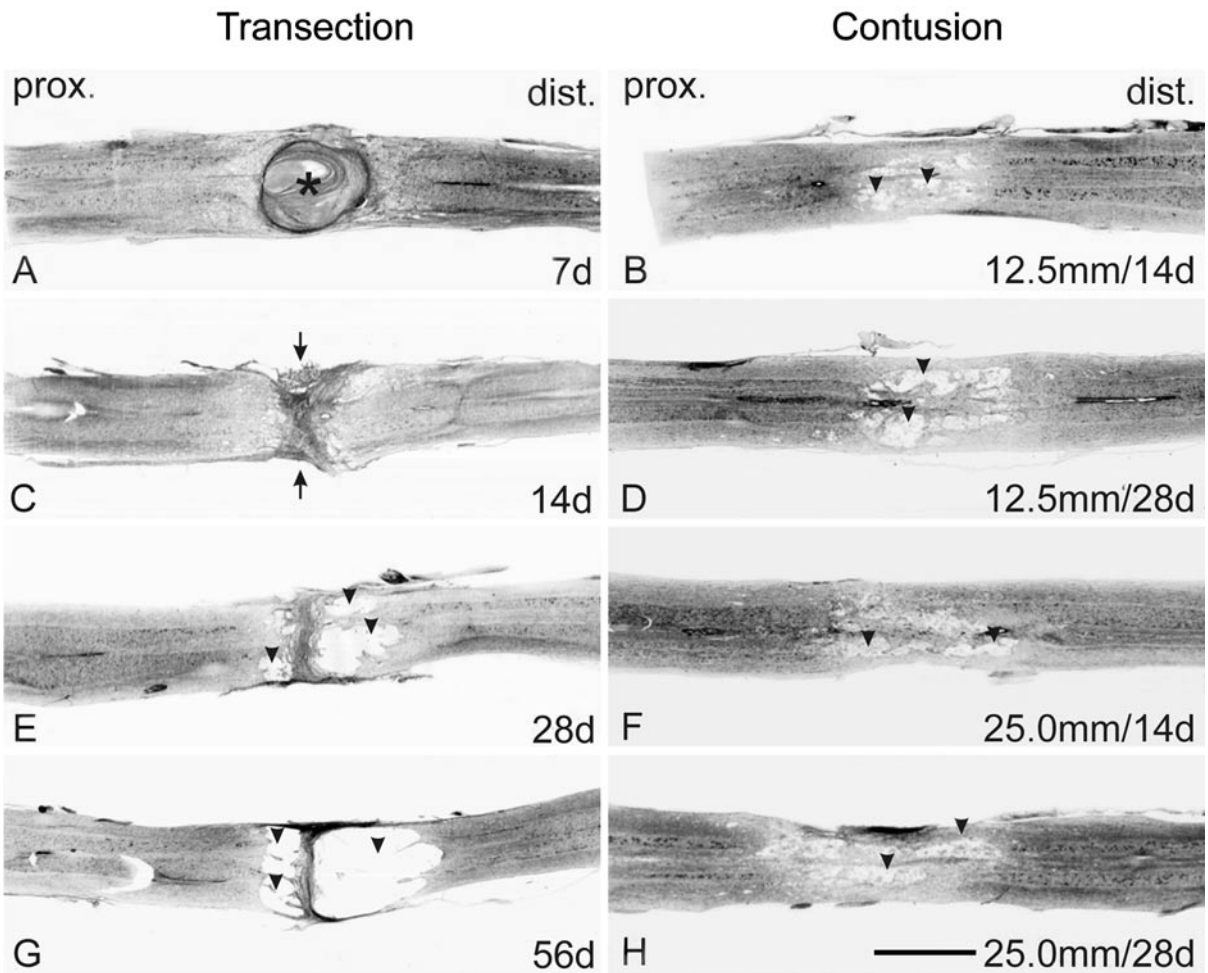

FIG. 1. Differences in lesion development in the adult rat spinal cord following complete transection or contusion lesion. Thionine stained horizontal sections through the rat spinal cord (T9) at 7, 14, 28, and 56 days after complete transection (A, C, E, and G, respectively). Complete spinal cord transection causes accumulation of blood at the lesion site, visible as a clot at 7 days postinjury (asterisk in A). At 14 days the blood clot is cleared and fibroblast-like cells have migrated into the lesion and form a scar that occupies the entire lesion site (arrows in C). Also the first signs of tissue loss are visible between the infiltrated fibroblasts and spinal cord stumps. Ongoing degeneration results in the formation of large cavities at 28 days (arrowheads in E) and 56 days (arrowheads in G) postinjury. Horizontal sections through the degenerating rat T9 spinal cord at 14 (B, F) and 28 days (D, H) following mild (B, D) and severe (F, H) contusion lesion. In contrast to transection lesions, thionine-stained sections through the contused spinal cord display a largely intact leptomeningeal sheet and no massive migration of fibroblast-like cells into the lesion (B, D, F, H). 12.5- and 25.0-mm weight drop lesions caused comparable tissue loss after 14 and 28 days, visible as numerous cavities in the lesioned area (arrow heads). Bar, 5.5 mm.

many of the infiltrated cells, which filled up the lesion site, formed strands that appeared to be connected to the proliferating meningeal sheet (Fig. 1C). Secondary degeneration extended into the distal and proximal spinal cord, resulting in the formation of cystic cavities between the neural scar and the remaining spinal cord tissue. Due to continuing secondary degeneration, the number and the size of the cysts increased, more so distally than proximally, in the subsequent 14 days (Fig. 1E). Severe tissue loss was visible after 56 days, when large cystic cavities have been formed on both sides of the neural scar (Fig. 1G). The cysts contained strands of cells attached to the scar and the meningeal sheet.

Contusion (Figs. 1B, 1D, 1F, and 1H). Weight drop lesions caused damage in all spinal cord layers at the primary lesion site. After 14 days (Figs. 1B and 1F), in both 12.5- and 25-mm weight drop-lesioned animals, extensive secondary degeneration was observed extending distally and proximally and resulting in numerous cysts. The meningeal sheet did not increase in thickness, as was observed following spinal cord transection. Twenty-eight days postlesion the secondary degeneration has extended further from the lesion site and the cysts have increased in size (Figs. 1D and 1H). Most of the meningeal sheet appeared unaffected by the lesion.

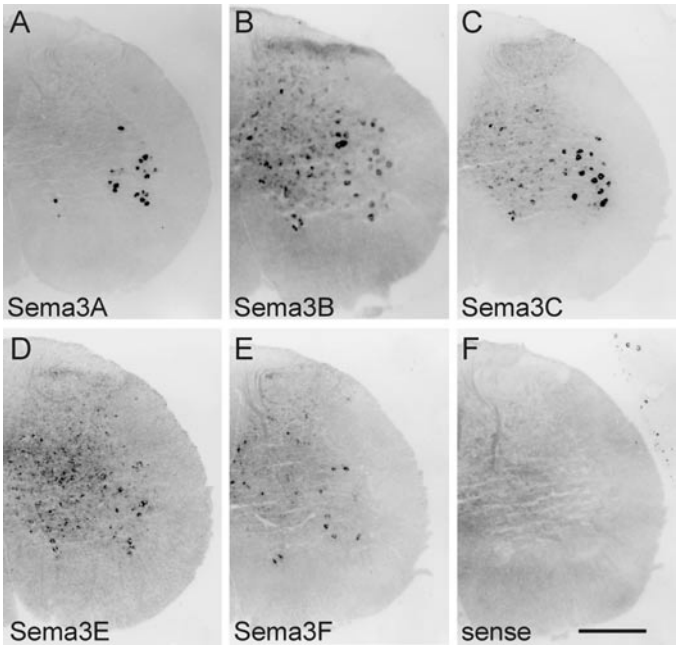

FIG. 2. Class 3 semaphorin expression in the non-injured rat spinal cord. *In situ* hybridization performed on transverse sections through the intact rat spinal cord. Expression of all class 3 semaphorins is restricted to neurons (A–E); no expression was observed in the white matter. Sema3A and Sema3C exhibited high expression levels in motor neurons (A, C), whereas Sema 3B, Sema3E, and Sema3F showed moderate expression levels (B, D, E) in motor neurons. A weak to moderate expression of Sema3B, Sema3C, Sema3E, and Sema3F was detected in a subpopulation of interneurons (B–F). Sections hybridized with Sema3A sense probe did not exhibit hybridization signals (F). Bar, 750 μm.

Class 3 Semaphorin Expression Following Transection and Contusion of the Spinal Cord

Since there are no antibodies available to detect class 3 semaphorin proteins in tissue sections, we used nonradioactive *in situ* hybridization to study transcript expression in horizontal spinal cord sections of completely transected and control rats. The spatiotemporal expression of class 3 semaphorin mRNA was analyzed at 7, 14, 28, and 56 days postlesion. In the intact adult rat spinal cord class 3 semaphorin expression is restricted to a subpopulation of neurons (Figs. 2A–2F). Occasionally however a weak expression of Sema3A and Sema3C in the meningeal sheet surrounding the spinal cord was observed (data not shown). Sections hybridized with sense probes exhibit no hybridization signal (Fig. 2F). Sema3B was expressed at high levels by cells located in both intact ventral and dorsal roots (Fig. 4F).

Spinal Cord Transection (Figs. 3A–3P). In the initial phase following transection class 3 semaphorin expression could not be detected at the lesion site (Figs. 3A–3D), only some of the spared neurons near the lesion site displayed class 3 semaphorins. At 14 days all members of the rodent class 3 semaphorins were abundantly expressed following complete spinal cord transection. Semaphorin 3A and 3C were expressed at high levels (Figs. 3E–3H), whereas 3B, 3E, and 3F were expressed at moderate levels. All class 3 semaphorins were expressed by the same type of elongated nonneuronal cells organized in string-like structures along the entire pial surface that surrounds the lesion site. These strands of semaphorin-positive cells were often connected to the swollen meningeal sheet surrounding the lesion site and extended deep into the lesion site. At 28 days, the expression has declined from high to moderate levels for Sema3A and 3C (Figs. 3I–3L) and from moderate to weak levels for Sema3B, 3E, and 3F. The semaphorin-positive cells were still most prominent along the pial border of the spinal cord lesion site. At the 56-day time point, Sema3A (Figs. 3M and 3N), 3B, 3E, and 3F could not be detected. Weak expression of Sema3C was present at the lateral edges of the lesion (Figs. 3O and 3P). The medial part of the lesion has turned into large cysts, filled with spinal fluid.

Spinal cord contusion (Figs. 3Q–3Z). Class 3 semaphorin mRNA expression was analyzed in rat thoracic spinal cord sections at 14 and 28 days following moderate (12.5-mm) and severe (25-mm) contusion injury, by performing *in situ* hybridization on horizontal sections through the lesion site. At both time points, moderate and severe contusion lesions resulted in comparable levels of semaphorin expression. Sema3A, 3B, and 3C showed high levels of expression (Figs. 3Q, 3R, 3U, 3V, 3Y, and 3Z), whereas 3E and 3F were moderately expressed (Figs. 3S, 3T, 3W, and 3X). Small scattered clusters of semaphorin-positive cells were positioned along or near the meningeal sheet surrounding the lesion site. The inner core of the lesion was mainly composed of cysts and contained sporadically semaphorin-positive cells. High Sema3B expression was not only observed in the meningeal sheet but also in cells located in the ventral and dorsal roots (Fig. 3U).

Characterization of the Semaphorin-Expressing Cell at the Transected Spinal Cord Lesion Site

Horizontal sections through the spinal lesion site (14 days postlesion) stained for individual class 3 semaphorin transcripts were subjected to immunohistochemistry using different markers for scar-associated cells to reveal the identity of the semaphorin-expressing cells in the developing scar. Complete transection of the spinal cord leads to a strong astrocytic reaction in and around the injury site. To determine whether class 3 semaphorins were expressed in astrocytes, the astrocyte marker GFAP was used to identify the astrocyte component of the scar. Double labeling with S100 was used to identify not only astrocytes, but also Schwann cells that could have migrated into the lesion. Antibodies to the intermediate filament protein vimen-

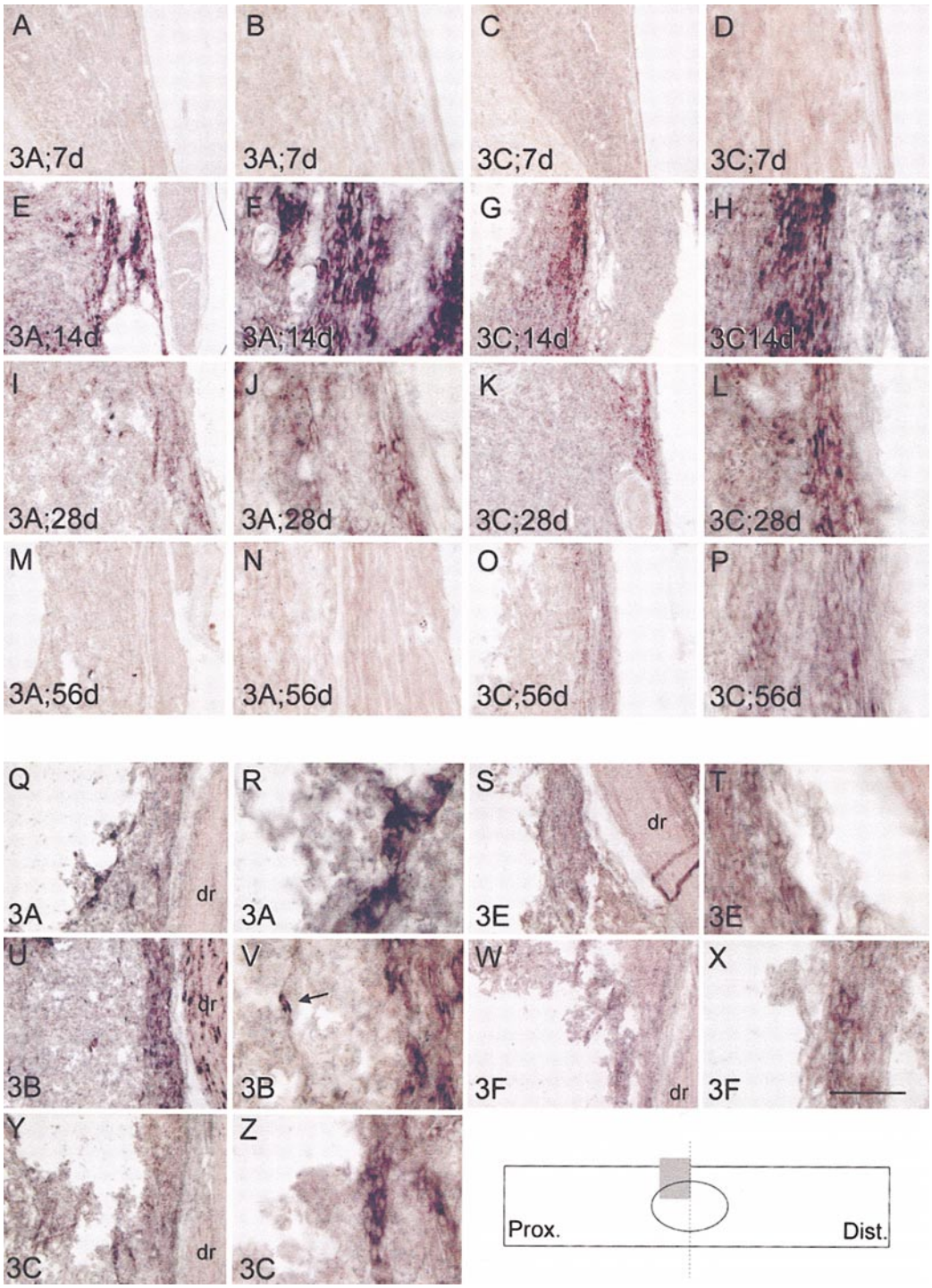

FIG. 3. Spatiotemporal distribution of class 3 semaphorins expressed in the transected and contused rat spinal cord. Bottom right, a schematic representation of a horizontal section through the rat spinal cord showing the approximate location of a complete transection (dotted line) or contusion lesion (oval). The gray box indicates the approximate position of the corresponding panels, all displayed with the surface of the spinal cord to the right. *In situ* hybridization performed on horizontal sections derived from the rat spinal cord transection lesions (A–P) and contusion lesions (Q–Z). Proximal is toward the top; B, D, F, H, J, L, N, P, R, T, V, X, and Z are higher magnifications of A, C, E, G, I, K, M, O, Q, S, U, W, and Y, respectively. Semaphorin 3A and 3C expression in the complete transected rat spinal cord at 7 days (A–D), 14 days (E–H), 28 days (I–L), and 56 days (M–P) survival time. Seven days following transection no mRNA encoding Sema3A was detected in the meningeal sheet surrounding the lesion site (A, B). Sema3C mRNA was very faintly present in the meningeal sheet at this time point (C, D). Strong induction of Sema3A (E, F) and Sema3C (K, L) expression in the next week resulted in heavily labeled cells in the meningeal sheet and in strings of cells connected to the meningeal sheet which extend deeper in the lesion at 14 days postinjury. Transcripts for Sema3A (I, J) and Sema3C (K, L) are continuously expressed at moderate levels at 28 days. At 56 days virtually no Sema3A and only weak

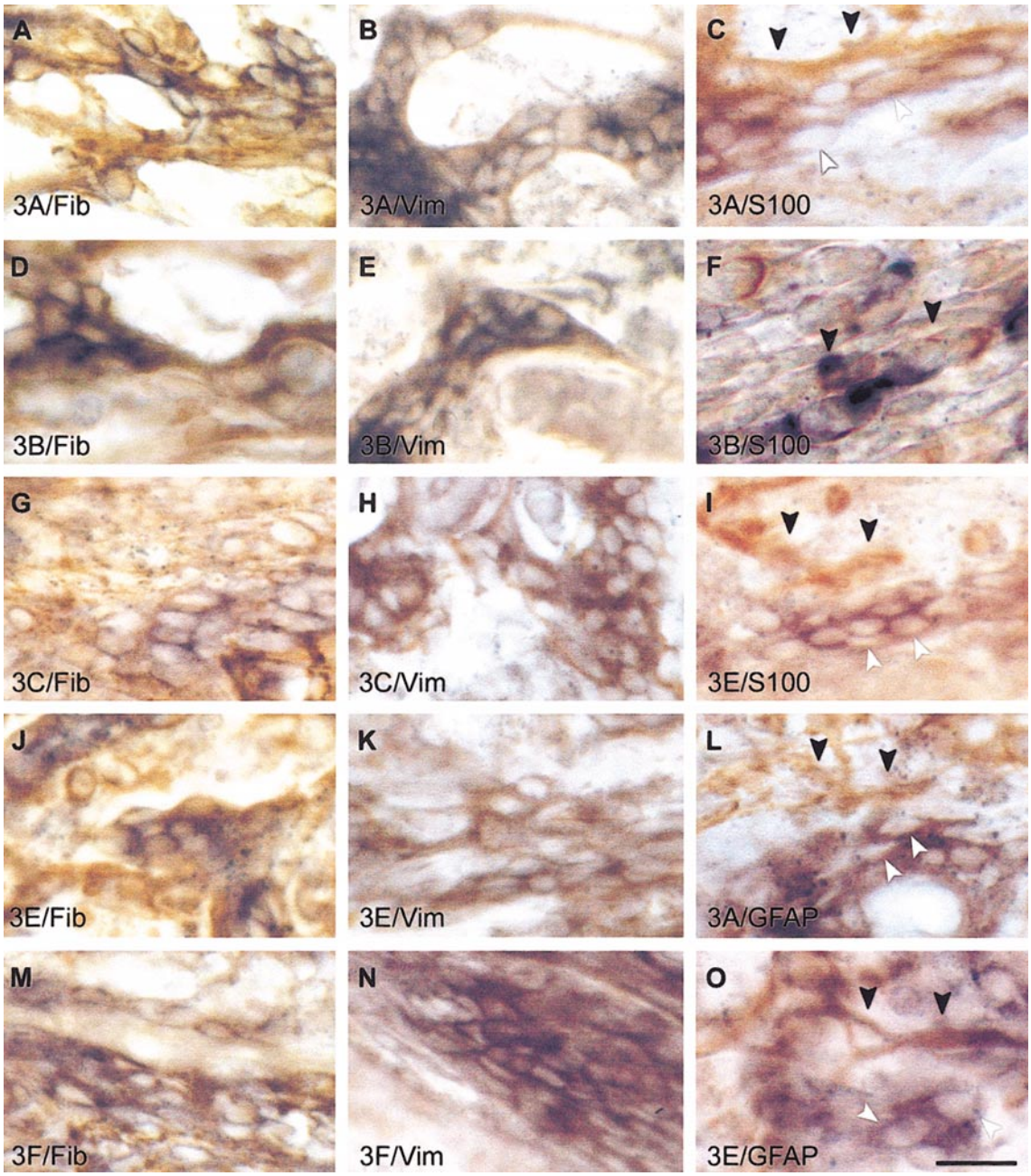

FIG. 4. Class 3 semaphorins are expressed by fibroblast-like cells in the injured spinal cord. Double labeling combining nonradioactive *in situ* hybridization for class 3 semaphorins (in purple) and immunohistochemistry (in brown) for fibronectin (A, D, G, J, M), vimentin (B, E, H, K, N), S100 (C, F, I), and GFAP (L, O) in sections through the spinal cord lesion site at 14 days following transection. Note that whereas double-labeled cells are dark brown (e.g., A, B), single-labeled profiles are either purple (e.g., C, L) or light brown (e.g., C, L). High-magnification shows that fibronectin colocalizes with messengers encoding the individual members of the class 3 semaphorins present in clustered cells throughout the lesion (A, D, G, J, M). Also the intermediate filament protein vimentin is expressed in clustered cells which are positive for one of the class 3 semaphorins (B, E, H, K, N). S100, a marker for astrocytes and Schwann cells (black arrowheads), does not colocalize with Sema3A and Sema3E transcripts (white arrowheads), whereas Sema3B-positive Schwann cells in the dorsal root do double-label with S-100 (black arrowheads) (F). GFAP-positive astrocytes (black arrowheads) were located in close proximity to Sema3A- and Sema3E-positive cells in the lesion core (white arrowheads). Bar, 20 μm.

Sema3C expression was observed in the remaining tissue. At 14 days following severe contusion of the adult rat spinal cord all rodent class 3 semaphorins are present at the lesion site. Sema3A (Q, R), Sema3B (U, V), and Sema3C (Y, Z) are expressed from moderate to strong levels, whereas Sema3E (S, T) and Sema3F (W, X) from moderate to weak levels. Most expression is observed in clusters of cells in or close to the meningeal sheet. Except for Sema3B (U), none of the class 3 semaphorins were detected in the dorsal roots (dr) (Q, Y, S, W). Strong Sema3B expression was also observed in cells with a Schwann cell-like phenotype deeper in the lesion site(arrow, V). Bar, 65 μm.

tin and to fibronectin were used to characterize the fibroblast component of the scar.

Following transection of the spinal cord strong GFAP expression could be observed at the borders of the lesion and in the lesion site itself. The clusters of semaphorin-expressing cells were located directly adjacent to GFAP-positive areas, but no colocalization was detected (Figs. 4L and 4O). Sema3A-, Sema3C-, Sema3E-, and Sema3F-expressing cells did not express S100 and showed a similar relation to S100 as to GFAP inside the lesion (Figs. 4C and 4I). Within the spinal roots, a subpopulation of S100-positive cells expressed Sema3B (Fig. 4F). Although most Sema3B-positive cells in the lesion were S100 negative, there was a small subpopulation of Sema3B-positive cells in the lesion that expressed S100; those are likely to represent Schwann cells that have migrated into the lesion site.

Fibronectin immunoreactivity was detected throughout the lesion site and was present in the relatively intact meningeal sheet more proximal and distal from the lesion site. Although the cellular localization of fibronectin was more widespread than that of the secreted semaphorins, all semaphorin-expressing cells in the lesion area were fibronectin positive (Figs. 4A, 4D, 4G, 4J, and 4M). Like fibronectin, vimentin could be detected throughout the neural scar and the meningeal sheet. All semaphorin-positive cells found in the scar were vimentin positive (Figs. 4B, 4E, 4H, 4K, and 4N). We therefore conclude that the semaphorin-expressing cells are meningeal fibroblasts.

Expression of NP-1, NP-2, PlxA1, and CRMP-2 by Cortico- and Rubrospinal Neurons

To determine if semaphorin expression at the spinal cord lesion site could repel damaged CST and RST axons, we performed *in situ* hybridization on coronal brain sections to detect the presence of semaphorin class 3 receptor components and CRMP-2 in intact and injured cortico- and rubrospinal neurons. The CST is formed by the axons of the pyramidal cells located in layer V of the motor cortex (39). In the uninjured adult animal, NP-1 transcripts were restricted to layer V neurons of the motor cortex (Fig. 5E). At 2 weeks following complete transection or contusion expression of NP-1 persists and additional weak NP-1 expression was observed in neurons of layer II and VI (Fig. 5A). Expression in these cortical layers could not be detected at later time points. In contrast to NP-1, NP-2, Plx1A, and CRMP-2 were widely expressed throughout the cortex of control animals, including layer V motor neurons (Figs. 5F–5H). Except for a small increase in NP-2 expression in layer II neurons at 14 days after complete spinal cord transection or contusion lesion, no changes in NP-2, PlxA1, and CRMP-2 mRNA expression levels were observed following spinal cord injury (Figs. 5B–5D).

In neurons of the red nucleus NP-1 mRNA could not be detected in either control or lesioned animals (figs. 6A and 6B). Transcripts of NP-2, PlxA1, and CRMP-2 were found in the magnocellular and parvocellular part of the red nucleus in both the control and the lesion situation (Figs. 6C and 6D, 6F and 6G, and 6H and 6I, respectively). Persistent expression of receptor components and CRMP-2 was observed up to 56 days postlesion. This indicates that axotomized CST and RST neurons could still respond to scar derived class 3 semaphorins.

Retrograde Tracing of the Descending Spinal Cord Fibers

To determine whether the neurons in layer V of the motor cortex containing semaphorin receptor components participate in the CST, we performed retrograde tracing combined with *in situ* hybridization for the receptor transcripts. Bilateral Fluorogold application in the dorsal half of the spinal cord at vertebra T6–7, followed by immunohistochemical detection of retrogradely transported Fluorogold in coronal brain sections, showed labeling of a subpopulation of layer V pyramidal cells. Combining immunohistochemical detection of Fluorogold with ISH for semaphorin class 3 receptor transcripts and CRMP-2 revealed dual staining in numerous cells in layer V of the motor cortex (Figs. 7A–7D). Double staining of coronal brain sections through the red nucleus of the same animals resulted in a subpopulation of cells which were Fluorogold-labeled and expressed transcripts for the semaphorin class 3 receptor components (data not shown). Expression of neuropilins, plexin A1, and CRMP-2 in Fluorogold-positive neurons indicates that neurons give rise to the CST and RST are likely to be able to sense scar-derived semaphorins.

Relation of the Descending Spinal Cord Fibers to the Neural Scar

To examine the relation between axotomized descending fibers and semaphorin-positive scar tissue following complete spinal cord transection, an anterograde tracer was injected into the proximal part of the lesioned cord. After 7 days horizontal sections through the lesion site were prepared and subjected to immunohistochemistry and *in situ* hybridization. At 7 days after complete transection of the spinal cord no semaphorin expression was detected at the lesion site. BDA-positive fibers could be detected in the proximal spinal cord stump, but did not reach the proximal border of the lesion (not shown). At 14 and 28 days following complete transection many traced fibers were detected in the proximal stump of the spinal cord, the majority of which appear to avoid the

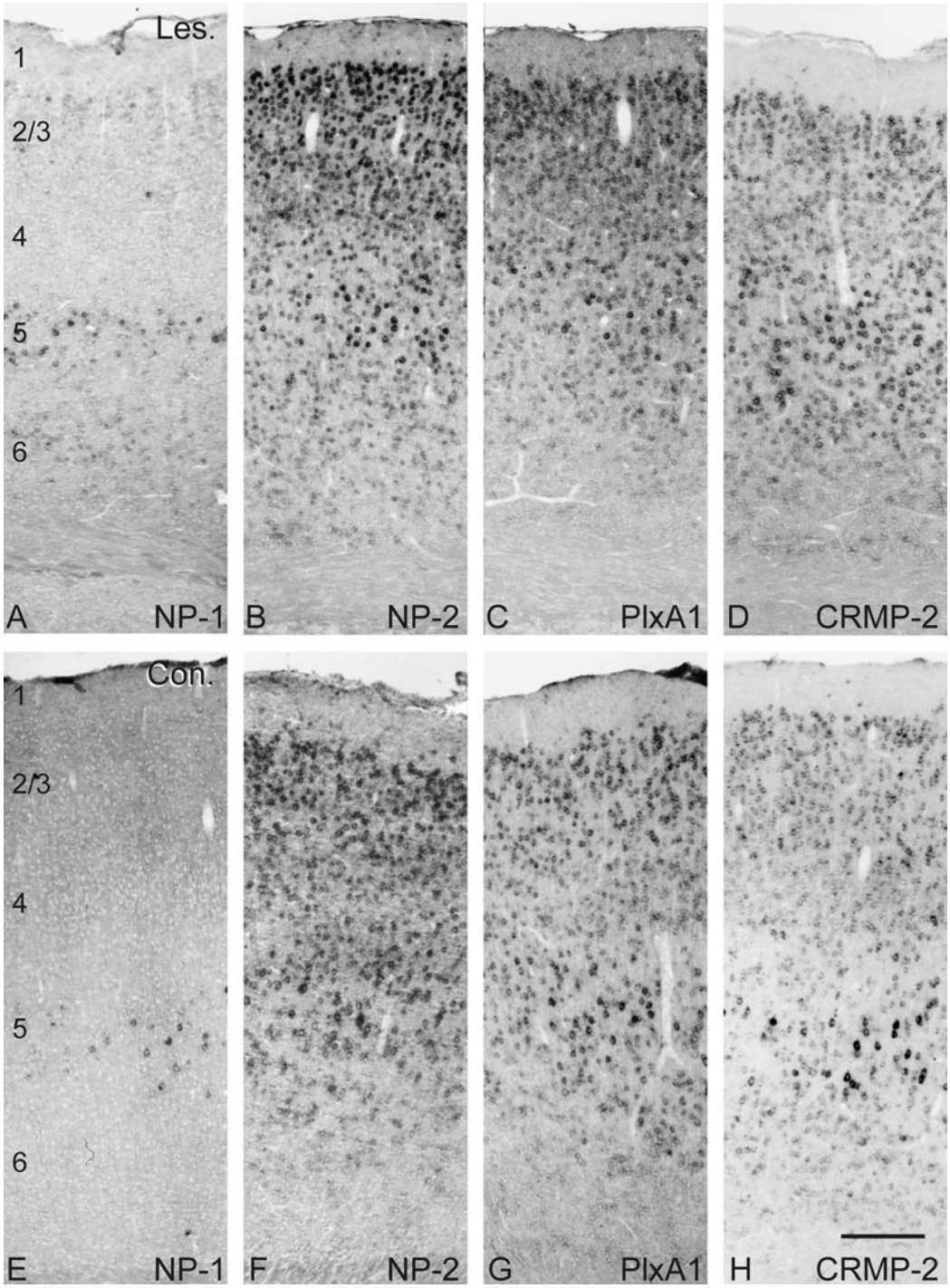

FIG. 5. Class 3 semaphorin receptor components are expressed by control and injured CST neurons. Transverse sections through motor cortex from control and spinal cord transected animals were subjected to *in situ* hybridization for NP-1 (A, E), NP-2 (B, F), PlxA1 (C, G), and CRMP-2 (D, H) mRNA. NP-1 is expressed in neurons located in layer V of the motor cortex in both control (E) and lesioned (A) animals. As a result of the spinal cord injury an additional staining for NP-1 mRNA was observed in neurons of layer II and VI of the motor cortex at 14 days postlesion (A). In contrast to NP-1, NP-2 (B, F), PlxA1(C, G), and CRMP-2 (D, H) mRNA are expressed in all cortical layers and by nearly all neurons. Except for a small increase of NP-2 expression in the neurons of layer II at 14 days after injury, no changes in PlxA1 and CRMP-2 expression were detected as a result of the lesion. Bar, 100 μm.

class 3 semaphorin-expressing parts in the scar (Fig. 7E). Occasionally, however, a BDA-labeled fiber penetrated deeply into the semaphorin-containing area (Fig. 7F). Due to tissue loss traced fibers near the scar were hard to detect 56 days after transection; the fibers that were visible were all located proxi-

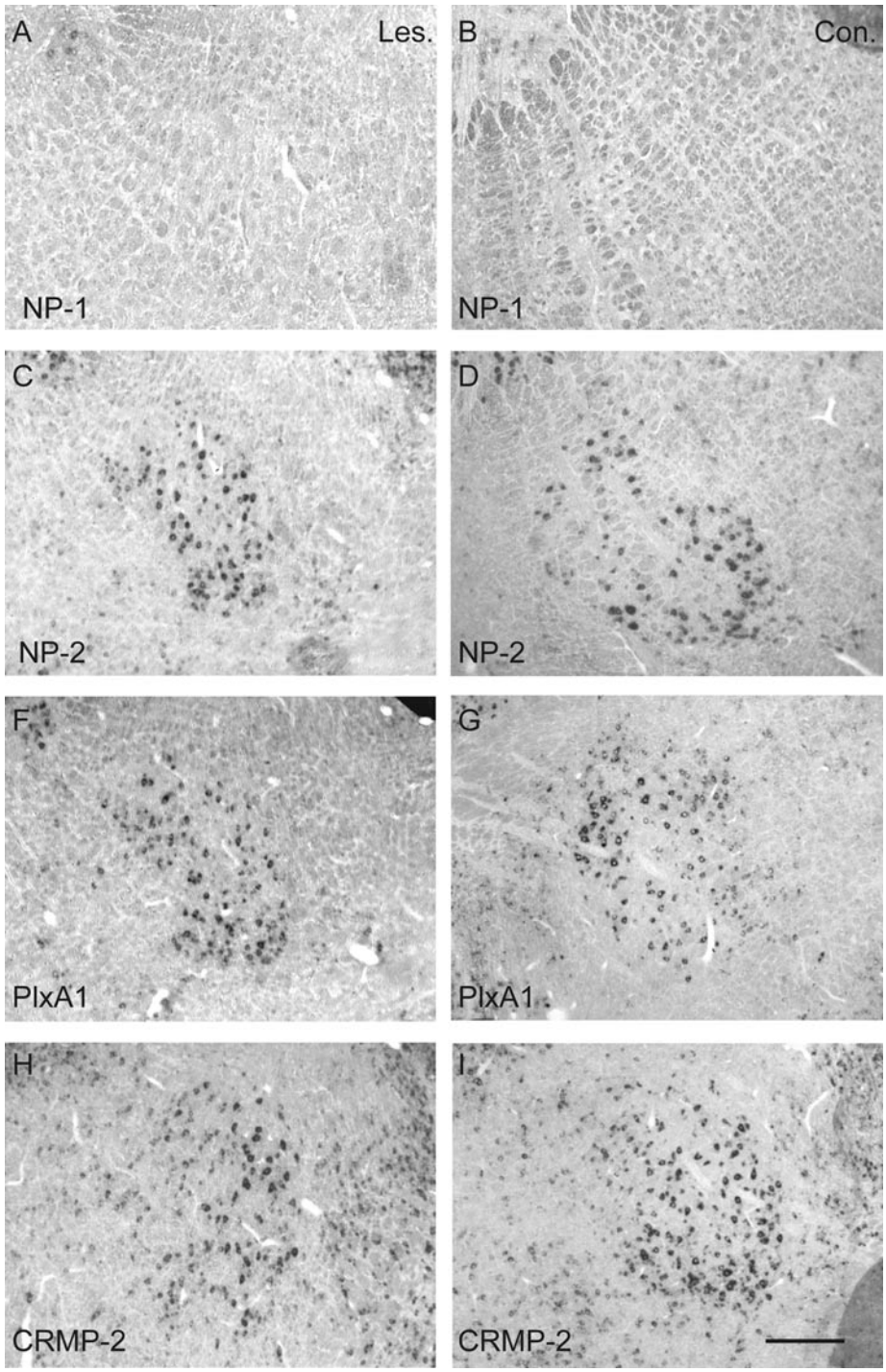

FIG. 6. Class 3 semaphorin receptor components are expressed by control and injured RST neurons. Transverse sections through the midbrain at the level of the red nucleus were subjected to in situ hybridization for NP-1 (A, B), NP-2 (C, D), PlxA1 (F, G), and CRMP-2 mRNA (H, I). NP-1 mRNA was not detected in the neurons of the red nucleus in the control situation and following spinal cord transection (A, B). NP-2, PlxA1, and CRMP-2 mRNAs are expressed by all rubrospinal neurons (D, G, and I, respectively), and the expression does not change following spinal cord transection (C, F, and H, respectively). Bar, 225 μm.

mally to the large cavities formed in the proximal spinal cord stump. Anterograde tracing of descending spinal cord fibers revealed that these injured spinal cord fibers did not grow across the lesion site and were unable to penetrate deeply into the semaphorin-positive portion of the scar.

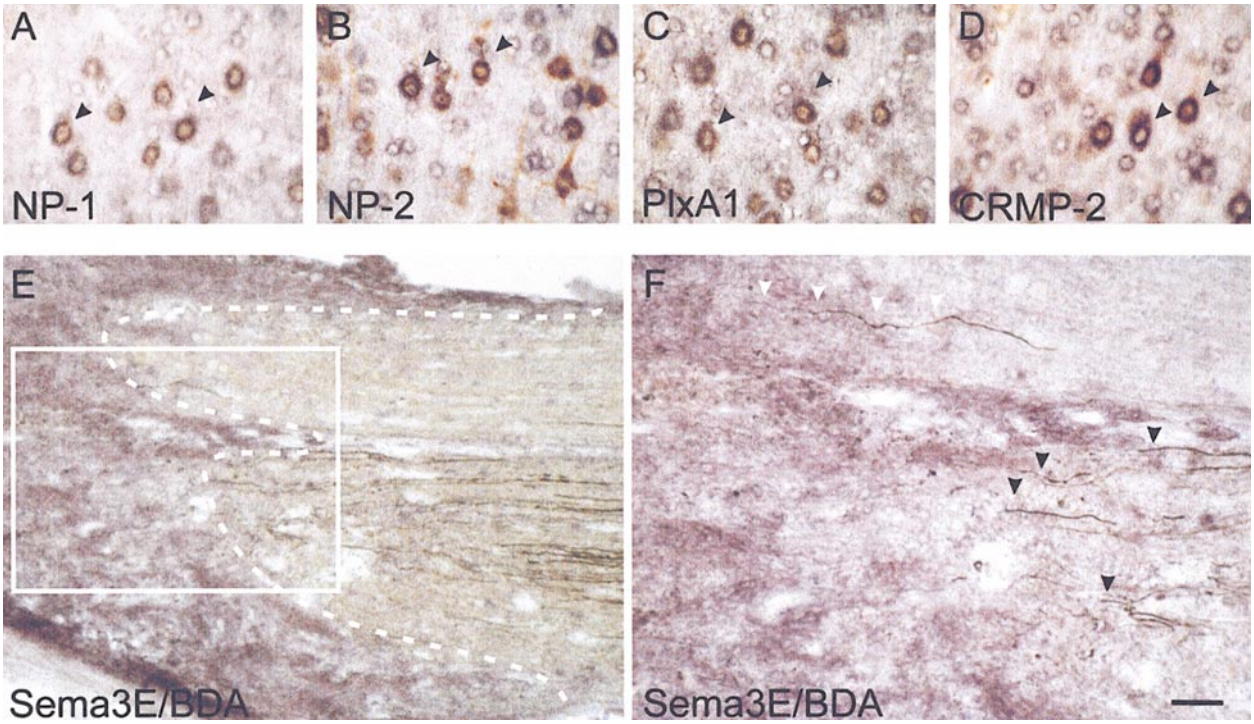

FIG. 7. (A–D) Retrograde traced CST neurons express class 3 semaphorin receptor components. Retrograde tracing combined with *in situ* hybridization on transversal sections through the rat motor cortex was used to reveal the presence of class 3 semaphorin receptor components in corticospinal tract forming layer V neurons (A–D). Black arrowheads point to neurons which were double labeled for Fluorogold (in brown) and one of the receptor components (in purple), indicating that NP-1, NP-2, PlxA1, and CRMP-2 are all expressed by the neurons that give rise to the corticospinal tract. Bar, 125 μm. (E and F) Relationship between injured descending spinal cord fibers and semaphorin 3E mRNA expression in the transected spinal cord. Horizontal sections of the spinal cord lesion site at 14 days after complete transection (E, boxed area in E is shown at F). Proximal is to right. Sections were probed for Sema3E (purple) and immunohistochemistry for BDA (dark brown). Dashed line indicates the interface between the semaphorin 3E-positive scar and the proximal spinal cord stump (E). Most anterogradely BDA-traced fibers (black arrowheads), present in the proximal spinal cord stump, fail to penetrate the semaphorin-positive portion of the scar (F). Occasional fibers do penetrate in the semaphorin 3E-expressing meningeal cells (white arrowheads in F). Bar, 400 μm in E; 210 μm in F.

DISCUSSION

In this study we show that following transection of the adult rat thoracic spinal cord the genes encoding Sema3A, 3B, 3C, 3E, and 3F are expressed by fibroblasts in the core of the scar and in the meningeal sheet surrounding the lesion site. After contusion lesion of the spinal cord, virtually no class 3 semaphorin expression was observed in the core of the scar, but semaphorin expression was induced in cells in the meningeal sheet surrounding the lesion site. Following spinal cord lesioning, the neurons that form the major descending spinal nerve tracts, the CST and RST, continue to express the components of class 3 semaphorin receptors, NP-1, NP-2, and PlxA1, and the intracellular signaling protein CRMP-2. Tracing studies revealed that virtually none of the descending spinal cord fibers enter semaphorin-positive regions of the glial–fibroblastic scar. Our observations indicate that chemorepulsive proteins of the semaphorin family may be involved in the failure of spinal cord axons to grow across scar tissue formed following spinal cord injury.

Spatiotemporal Distribution of Class 3 Semaphorin Expression in the Injured Spinal Cord

Transection and contusion lesions of the spinal cord are two commonly used experimental models to study the failure of axonal regeneration and glial scar formation. Following both types of lesion we observe an induction in expression of all known members of the class 3 semaphorins in small spindle-shaped cells with elongated nuclei. Double labeling studies show that cells expressing class 3 semaphorins are coexpressed with markers for fibroblasts, including fibronectin (56) and vimentin (43), and do not express the astrocyte marker GFAP (8). These data are consistent with and extend previous observations showing that the prototypic member of the class 3 semaphorins, Sema3A, is expressed in fibroblasts derived from the leptomeningeal sheet after injury to the adult CNS (46, 47). Sema3B, but none of the other class 3 semaphorins, is also expressed in S100-positive/GFAP-negative cells in the dorsal and ventral roots and in cells located in the lesion site. This indicates that Sema3B-expressing

S100-positive cells are Schwann cells that have migrated into the spinal cord lesion. These migrated Schwann cells are most likely derived from the dorsal and ventral roots (38), where cells with high levels of Sema3B expression were also observed. This is in line with the expression of Sema3B in Schwann cell precursors during embryonic development (50).

Interestingly, the distribution of semaphorin-positive fibroblasts differs markedly in scars formed after transection or contusion lesion. Previous morphological studies have revealed profound differences in the response of the meningeal sheet surrounding the lesion site following spinal cord transection or contusion lesion. Whereas penetrating injuries are characterized by proliferation and migration of meningeal fibroblasts into the lesion (14, 34), this does not occur after contusion lesions. Contusion lesions leave the meningeal sheet largely intact and a spared rim of white matter remains present. This probably prevents massive proliferation and migration of meningeal fibroblasts deep into the lesion (6, 9). In line with this, spinal cord transection results in abundant class 3 semaphorin expression in the meningeal sheet and in strings and patches of fibroblast-like cells extending deep into the center of the lesion. Following contusion lesion, however, class 3 semaphorin expression is restricted to cells of the meningeal sheet surrounding the injury site. Thus, the distribution of class 3 semaphorin-positive cells appears to be consistent with the lesion-induced response displayed by meningeal fibroblasts.

In line with the minor cellular reaction of the meningeal sheet during the first week following spinal cord injury no class 3 semaphorin expression was detected in the lesion and the surrounding meningeal sheet during the first postlesion period. In the second week following transection an increase in thickness of the meningeal sheet was accompanied by robust expression of all class 3 semaphorin genes in meningeal fibroblasts. The progressive degeneration of spinal cord tissue due to ischemia and edema results in the formation of large cavities at 56 days postlesion (51, 68). With the exception of some faintly Sema3C-positive cells, no class 3 semaphorin-positive meningeal fibroblasts were detected at 56 days posttransection, probably as a direct consequence of cavity formation and tissue loss.

Class 3 Semaphorin Receptor Components Are Expressed by Intact and Injured CST and RST Motor Neurons

Combined retrograde tracing and *in situ* hybridization revealed the expression of class 3 semaphorin receptor components and the intracellular signaling molecule CRMP-2 in neurons that give rise to the CST and RST. Although spinal cord transection caused no changes in receptor complex expression in neurons affected by the injury, spinal cord transection induced NP-1 expression in layers II and VI of the motor cortex. NP-2 gene expression was also slightly increased in layer II. It is not clear if these changes are related to alterations in synaptic contacts of layer V neurons on layer II/VI neurons, or whether they are due to increased fore paw use, which changes the sensory input in superficial cortex layers and effects dendritic branching (52). Previous studies have also observed that spinal cord transection induces changes in the expression of other genes in cortical layers other then layer V. For example, increased expression of the transcription factor c-*fos* was found in neurons of all cortical layers (24).

Descending Axons Fail to Penetrate the Semaphorin-Positive Component of the Scar

Cortico- and rubrospinal neurons are able to form sprouts near the lesion site but these neurons do not regrow axons across the injury site (35). Anterograde tracing of descending fibers with BDA in combination with *in situ* hybridization for the secreted semaphorins allowed us to study the relationship between injured axon endings and semaphorin-positive cells in the scar. The majority of BDA-positive fibers did not penetrate semaphorin class 3-positive portions of the neural scar following transection. During development cortical axons are known to be responsive to the repulsive actions of Sema3A, 3B, and 3E present in the developing spinal cord (10, 48, 49). Previous studies showed the avoidance of Sema3A-positive meningeal cells in the scar by regenerating, NP-1-positive, dorsal column fibers in the neural scar formed following transection lesions (45). Furthermore, fibers of transected primary olfactory neurons did not grow across Sema3A-expressing fibroblasts that fill up the bulbar cavity after olfactory bulbectomy (46). The persistent expression of the receptor components by axons of the two major descending tracts and the failure of most descending axons to enter the semaphorin-positive portion of the scar suggests that secreted semaphorins contribute to the inhibitory nature of CNS scar tissue.

The observation that occasionally axons do grow deeply into the semaphorin-positive regions of the injured spinal cord may be explained by the fact that not all descending fibers may be responsive to scar-derived semaphorins. Since we traced all descending fibers it is well possible that those axons originate from other sources than CST or RST neurons and may lack essential receptor components or secondary messengers required to sense class 3 semaphorins.

Our results show an increased expression of class 3 semaphorins in meningeal fibroblasts at the spinal cord lesion site. Interestingly, the temporal expression of class 3 semaphorins coincides with the period of growth arrest of injured CST and RST fibers. This

suggests that the migration of semaphorin-positive fibroblasts into the lesion site could contribute to the formation of a molecular barrier preventing regenerating fibers from penetrating the distal spinal cord. Further analysis of the role of secreted semaphorins in neural scar formation and their contribution to the inhibitory nature of the CNS scar could provide valuable insights in the need to neutralize these proteins. Antibody blocking of class 3 semaphorins or overexpression of dominant negative receptors on damaged axons might be an essential part of a multiapproach strategy for creating a more permissive environment for axonal regeneration in the injured spinal cord.

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REFERENCES

1. Semaphorin Nomenclature Committee. 1999. Unified nomenclature for the semaphorins/collapsins. *Cell* **97:** 551–552.
2. Ajemian, A., Ness, R., and David, S. 1994. Tenascin in the injured rat optic nerve and in non-neuronal cells in vitro: Potential role in neural repair. *J. Comp. Neurol.* **340:** 233–242.
3. Anderson, P. N. 1999. *Intrinsic Determinants of Differential Axonal Regeneration by Adult Mammalian CNS Neurons.* Harwood Academic, Reading, UK.
4. Aubert, I., J. L. Ridet, and F. H. Gage. 1995. Regeneration in the adult mammalian CNS: Guided by development. *Curr. Opin. Neurobiol.* **5:** 625–635.
5. Bahr, M., and F. Bonhoeffer. 1994. Perspectives on axonal regeneration in the mammalian CNS. *Trends Neurosci.* **17:** 473–479.
6. Basso, D. M., M. S. Beattie, and J. C. Bresnahan. 1995. A sensitive and reliable locomotor rating scale for open field testing in rats. *J. Neurotrauma* **12:** 1–21.
7. Behar, O., J. A. Golden, H. Mashimo, F. J. Schoen, and M. C. Fishman. 1996. Semaphorin III is needed for normal patterning and growth of nerves, bones and heart. *Nature* **383:** 525–528.
8. Bignami, A., and D. Dahl. 1974. Astrocyte-specific protein and radial glia in the cerebral cortex of newborn rat. *Nature* **252:** 55–56.
9. Caroni, P., and M. E. Schwab. 1988. Two membrane protein fractions from rat central myelin with inhibitory properties for neurite growth and fibroblast spreading. *J. Cell Biol.* **106:** 1281–1288.
10. Castellani, V., A. Chedotal, M. Schachner, C. Faivre-Sarrailh, and G. Rougon. 2000. Analysis of the L1-deficient mouse phenotype reveals cross-talk between Sema3A and L1 signaling pathways in axonal guidance. *Neuron* **27:** 237–249.
11. Chen, H., A. Chedotal, Z. He, C. S. Goodman, and M. Tessier-Lavigne. 1997. Neuropilin-2, a novel member of the neuropilin family, is a high affinity receptor for the semaphorins Sema E and Sema IV but not Sema III. *Neuron* **19:** 547–559.
12. Cole, G. J., and C. F. McCabe. 1991. Identification of a developmentally regulated keratan sulfate proteoglycan that inhibits cell adhesion and neurite outgrowth. *Neuron* **7:** 1007–1018.
13. Dottori, M., L. Hartley, M. Galea, G. Paxinos, M. Polizzotto, T. Kilpatrick, P. F. Bartlett, M. Murphy, F. Kontgen, and A. W. Boyd. 1998. EphA4 (Sek1) receptor tyrosine kinase is required for the development of the corticospinal tract. *Proc. Natl. Acad. Sci. USA* **95:** 13248–13253.
14. Fawcett, J. W., and R. A. Asher. 1999. The glial scar and central nervous system repair. *Brain Res. Bull.* **49:** 377–391.
15. Feiner, L., A. M. Koppel, H. Kobayashi, and J. A. Raper. 1997. Secreted chick semaphorins bind recombinant neuropilin with similar affinities but bind different subsets of neurons in situ. *Neuron* **19:** 539–545.
16. Frisen, J., P. A. Yates, T. McLaughlin, G. C. Friedman, D. D. O'Leary, and M. Barbacid. 1998. Ephrin-A5 (AL-1/RAGS) is essential for proper retinal axon guidance and topographic mapping in the mammalian visual system. *Neuron* **20:** 235–243.
17. Giger, R. J., J. F. Cloutier, A. Sahay, R. K. Prinjha, D. V. Levengood, S. E. Moore, S. Pickering, D. Simmons, S. Rastan, F. S. Walsh, A. L. Kolodkin, D. D. Ginty, and M. Geppert. 2000. Neuropilin-2 is required in vivo for selective axon guidance responses to secreted semaphorins. *Neuron* **25:** 29–41.
18. Giger, R. J., R. J. Pasterkamp, S. Heijnen, A. J. Holtmaat, and J. Verhaagen. 1998. Anatomical distribution of the chemorepellent semaphorin III/collapsin-1 in the adult rat and human brain: Predominant expression in structures of the olfactory–hippocampal pathway and the motor system. *J. Neurosci. Res.* **52:** 27–42.
19. Giger, R. J., D. P. Wolfer, G. M. De Wit, and J. Verhaagen. 1996. Anatomy of rat semaphorin III/collapsin-1 mRNA expression and relationship to developing nerve tracts during neuroembryogenesis. *J. Comp. Neurol.* **375:** 378–392.
20. Goshima, Y., F. Nakamura, P. Strittmatter, and S. M. Strittmatter. 1995. Collapsin-induced growth cone collapse mediated by an intracellular protein related to UNC-33. *Nature* **376:** 509–514.
21. Gruner, J. A. 1992. A monitored contusion model of spinal cord injury in the rat. *J. Neurotrauma* **9:** 123–126; discussion 126–128.
22. Guthrie, S. 1999. Axon guidance: Starting and stopping with slit. *Curr. Biol.* **9:** R432–R435.
23. He, Z., and M. Tessier-Lavigne. 1997. Neuropilin is a receptor for the axonal chemorepellent semaphorin III. *Cell* **90:** 739–751.
24. Ikeda, S., and S. Nakagawa. 1998. Spinal cord transection induced c-fos protein in the rat motor cortex. *Brain Res.* **792:** 164–167.
25. Jin, Z., and S. M. Strittmatter. 1997. Rac1 mediates collapsin-1-induced growth cone collapse. *J. Neurosci.* **17:** 6256–6263.
26. Kapfhammer, J. P. 1997. Axon sprouting in the spinal cord: Growth promoting and growth inhibitory mechanisms. *Anat. Embryol. (Berlin)* **196:** 417–426.
27. Kolodkin, A. L. 1996. Growth cones and the cues that repel them. *Trends Neurosci.* **19:** 507–513.
28. Kolodkin, A. L., D. V. Levengood, E. G. Rowe, Y. T. Tai, R. J. Giger, and D. D. Ginty. 1997. Neuropilin is a semaphorin III receptor. *Cell* **90:** 753–762.
29. Kolodkin, A. L., D. J. Matthes, and C. S. Goodman. 1993. The semaphorin genes encode a family of transmembrane and secreted growth cone guidance molecules. *Cell* **75:** 1389–1399.
30. Kuhn, T. B., M. D. Brown, C. L. Wilcox, J. A. Raper, and J. R. Bamburg. 1999. Myelin and collapsin-1 induce motor neuron growth cone collapse through different pathways: Inhibition of

collapse by opposing mutants of rac1. *J. Neurosci.* **19:** 1965–1975.

31. Lankhorst, A. J., S. E. Duis, M. P. ter Laak, E. A. Joosten, F. P. Hamers, and W. H. Gispen. 1999. Functional recovery after central infusion of alpha-melanocyte-stimulating hormone in rats with spinal cord contusion injury. *J. Neurotrauma* **16:** 323–331.
32. Laywell, E. D., U. Dorries, U. Bartsch, A. Faissner, M. Schachner, and D. A. Steindler. 1992. Enhanced expression of the developmentally regulated extracellular matrix molecule tenascin following adult brain injury. *Proc. Natl. Acad. Sci. USA* **89:** 2634–2638.
33. Laywell, E. D., and D. A. Steindler. 1991. Boundaries and wounds, glia and glycoconjugates: Cellular and molecular analyses of developmental partitions and adult brain lesions. *Ann. N. Y. Acad. Sci.* **633:** 122–141.
34. Li, M. S., and David, S. 1996. Topical glucocorticoids modulate the lesion interface after cerebral cortical stab wounds in adult rats. *Glia* **18:** 306–318.
35. Li, Y., and G. Raisman. 1995. Sprouts from cut corticospinal axons persist in the presence of astrocytic scarring in long-term lesions of the adult rat spinal cord. *Exp. Neurol.* **134:** 102–111.
36. Lips, K., C. C. Stichel, and H. W. Muller. 1995. Restricted appearance of tenascin and chondroitin sulphate proteoglycans after transection and sprouting of adult rat postcommissural fornix. *J. Neurocytol.* **24:** 449–464.
37. Luo, Y., D. Raible, and J. A. Raper. 1993. Collapsin: A protein in brain that induces the collapse and paralysis of neuronal growth cones. *Cell* **75:** 217–227.
38. Malhotra, S. K., T. K. Shnitka, and J. Elbrink. 1990. Reactive astrocytes—A review. *Cytobios* **61:** 133–160.
39. McKeon, R. J., A. Hoke, and J. Silver. 1995. Injury-induced proteoglycans inhibit the potential for laminin-mediated axon growth on astrocytic scars. *Exp. Neurol.* **136:** 32–43.
40. McKerracher, L., S. David, D. L. Jackson, V. Kottis, R. J. Dunn, and P. E. Braun. 1994. Identification of myelin-associated glycoprotein as a major myelin-derived inhibitor of neurite growth. *Neuron* **13:** 805–811.
41. Miranda, J. D., L. A. White, A. E. Marcillo, C. A. Willson, J. Jagid, and S. R. Whittemore. 1999. Induction of Eph B3 after spinal cord injury. *Exp. Neurol.* **156:** 218–222.
42. Mukhopadhyay, G., P. Doherty, F. S. Walsh, P. R. Crocker, and M. T. Filbin. 1994. A novel role for myelin-associated glycoprotein as an inhibitor of axonal regeneration. *Neuron* **13:** 757–767.
43. Osborn, M., E. Debus, and K. Weber. 1984. Monoclonal antibodies specific for vimentin. *Eur. J. Cell Biol.* **34:** 137–143.
44. Oudega, M., C. G. Vargas, A. B. Weber, N. Kleitman, and M. B. Bunge. 1999. Long-term effects of methylprednisolone following transection of adult rat spinal cord. *Eur. J. Neurosci.* **11:** 2453–2464.
45. Pasterkamp, R. J., P. N. Anderson, and J. Verhaagen. 2001. Peripheral nerve injury fails to induce growth of lesioned ascending dorsal column axons into spinal cord scar tissue expressing the axon repellent Semaphorin3A. *Eur. J. Neurosci.* **13:** 457–471.
46. Pasterkamp, R. J., F. De Winter, R. J. Giger, and J. Verhaagen. 1998. Role for semaphorin III and its receptor neuropilin-1 in neuronal regeneration and scar formation? *Prog. Brain Res.* **117:** 151–170.
47. Pasterkamp, R. J., R. J. Giger, M. J. Ruitenberg, A. J. Holtmaat, J. De Wit, F. De Winter, and J. Verhaagen. 1999. Expression of the gene encoding the chemorepellent semaphorin III is induced in the fibroblast component of neural scar tissue formed following injuries of adult but not neonatal CNS. *Mol. Cell. Neurosci.* **13:** 143–166.
48. Polleux, F., R. J. Giger, D. D. Ginty, A. L. Kolodkin, and A. Ghosh. 1998. Patterning of cortical efferent projections by semaphorin–neuropilin interactions. *Science* **282:** 1904–1906.
49. Polleux, F., T. Morrow, and A. Ghosh. 2000. Semaphorin 3A is a chemoattractant for cortical apical dendrites. *Nature* **404:** 567–573.
50. Puschel, A. W., R. H. Adams, and H. Betz. 1996. The sensory innervation of the mouse spinal cord may be patterned by differential expression of and differential responsiveness to semaphorins. *Mol. Cell. Neurosci.* **7:** 419–431.
51. Quencer, R. M., and R. P. Bunge. 1996. The injured spinal cord: Imaging, histopathologic clinical correlates, and basic science approaches to enhancing neural function after spinal cord injury. *Spine* **21:** 2064–2066.
52. Rao, B. S., T. Desiraju, and T. R. Raju. 1993. Neuronal plasticity induced by self-stimulation rewarding experience in rats—A study on alteration in dendritic branching in pyramidal neurons of hippocampus and motor cortex. *Brain Res.* **627:** 216–224.
53. Reier, P. J., and J. D. Houle. 1988. The glial scar: Its bearing on axonal elongation and transplantation approaches to CNS repair. *Adv. Neurol.* **47:** 87–138.
54. Rohm, B., A. Ottemeyer, M. Lohrum, and A. W. Puschel. 2000. Plexin/neuropilin complexes mediate repulsion by the axonal guidance signal semaphorin 3A. *Mech. Dev.* **93:** 95–104.
55. Rohm, B., B. Rahim, B. Kleiber, I. Hovatta, and A. W. Puschel. 2000. The semaphorin 3A receptor may directly regulate the activity of small GTPases. *FEBS Lett.* **486:** 68–72.
56. Ruoslahti, E., E. G. Hayman, M. Pierschbacher, and E. Engvall. 1982. Fibronectin: Purification, immunochemical properties, and biological activities. *Methods Enzymol.* **82:** 803–831.
57. Schnell, L., and M. E. Schwab. 1990. Axonal regeneration in the rat spinal cord produced by an antibody against myelin-associated neurite growth inhibitors. *Nature* **343:** 269–272.
58. Serafini, T., S. A. Colamarino, E. D. Leonardo, H. Wang, R. Beddington, W. C. Skarnes, and M. Tessier-Lavigne. 1996. Netrin-1 is required for commissural axon guidance in the developing vertebrate nervous system. *Cell* **87:** 1001–1014.
59. Snow, D. M., V. Lemmon, D. A. Carrino, A. I. Caplan, and J. Silver. 1990. Sulfated proteoglycans in astroglial barriers inhibit neurite outgrowth *in vitro*. *Exp. Neurol.* **109:** 111–130.
60. Snow, D. M., and P. C. Letourneau. 1992. Neurite outgrowth on a step gradient of chondroitin sulfate proteoglycan (CS-PG). *J. Neurobiol.* **23:** 322–336.
61. Steindler, D. A. 1993. Glial boundaries in the developing nervous system. *Annu. Rev. Neurosci.* **16:** 445–470.
62. Stichel, C. C., and H. W. Muller. 1995. Regenerative failure in the mammalian CNS. *Trends Neurosci* **18:** 128; discussion 128–129.
63. Stichel, C. C., and H. W. Muller. 1998. The CNS lesion scar: New vistas on an old regeneration barrier. *Cell Tissue Res* **294:** 1–9.
64. Takahashi, T., A. Fournier, F. Nakamura, L. H. Wang, Y. Murakami, R. G. Kalb, H. Fujisawa, and S. M. Strittmatter. 1999. Plexin-neuropilin-1 complexes form functional semaphorin-3A receptors. *Cell* **99:** 59–69.
65. Takahashi, T., F. Nakamura, Z. Jin, R. G. Kalb, and S. M. Strittmatter. 1998. Semaphorins A and E act as antagonists of neuropilin-1 and agonists of neuropilin-2 receptors. *Nature Neurosci.* **1:** 487–493.
66. Tamagnone, L., S. Artigiani, H. Chen, Z. He, G. I. Ming, H. Song, A. Chedotal, M. L. Winberg, C. S. Goodman, M. Poo, M. Tessier-Lavigne, and P. M. Comoglio. 1999. Plexins are a large family of receptors for transmembrane, secreted, and GPI-anchored semaphorins in vertebrates. *Cell* **99:** 71–80.

67. Taniguchi, M., S. Yuasa, H. Fujisawa, I. Naruse, S. Saga, M. Mishina, and T. Yagi. 1997. Disruption of semaphorin III/D gene causes severe abnormality in peripheral nerve projection. *Neuron* **19:** 519–530.

68. Tator, C. H. 1995. Update on the pathophysiology and pathology of acute spinal cord injury. *Brain Pathol.* **5:** 407–413.

69. Tessier-Lavigne, M., and C. S. Goodman. 1996. The molecular biology of axon guidance. *Science* **274:** 1123–1133.

70. Verhaagen, J., W. T. Hermens, P. A. Dijkhuizen, A. J. Holtmaat, and W. H. Gispen. 1995. Use of viral vectors to promote neuroregeneration. *Clin. Neurosci.* **3:** 275–283.

71. Zhang, Y., P. N. Anderson, G. Campbell, H. Mohajeri, M. Schachner, and A. R. Lieberman. 1995. Tenascin-C expression by neurons and glial cells in the rat spinal cord: changes during postnatal development and after dorsal root or sciatic nerve injury. *J. Neurocytol.* **24:** 585–601.